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Development and characterization of mouse monoclonal antibodies reactive with chicken CXCLi2

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ABSTRACT

Interleukin-8(IL-8)/CXCL8 is a CXC-family chemokine that attracts lymphocytes to sites of tissue damage and plays a role in the inflammatory response and wound healing. Chicken chemotactic and angiogenic factor was referred to as chCXCLi2 and has been studied as one of human CXCL8 homologue for more than 20 years. However, no monoclonal antibodies (mAbs) that specifically detect chCXCLi2 have been developed. Here, we developed and characterized mouse mAbs against chCXCLi2 to define its immunological properties. Two mouse mAbs against chCXCLi2 were generated and confirmed to display specific binding with not only recombinants, but endogenous chCXCLi2 by Western blot analysis, ELISA, and immunocytochemistry. Inhibition of chCXCLi2-induced chemotactic activity on peripheral blood lymphocytes, proliferation of chicken macrophage cells and expression of alpha smooth-muscle actin in chicken embryonic fibroblast cells by antibodies indicate that these antibodies are capable of blocking chCXCLi2 bioactivity. These chCXCLi2 mAbs will be useful reagents for future investigations of inflammation in poultry.

1. Introduction

The CXC chemokine family consists of two subfamilies, delineated by the presence or absence of the tripeptide Glu-Leu-Arg (ELR) motif located in the NH_2-terminal region and designated as ELR^+ or ELR^- CXC chemokines, respectively (Fernandez and Lolis, 2002). CXCL8, also known as interleukin-8 (IL-8), belongs to the ELR^+ CXC chemokine subfamily and functions as a neutrophil chemoattractant and potent angiogenic factor (Modi et al., 1990). Additionally, CXCL8 plays an important role in inflammation and wound healing. Elevated IL-8 expression and neutrophil infiltration are observed in many inflammatory and recovery responses (Bickel, 1993; Heidemann et al., 2003).

For chicken homologues of human CXCL8 (huCXCL8), 9E3 (Sugano et al., 1987) and pCEF-4 (Bedard et al., 1987) (referred to as chCXCLi2 by Kaiser et al. (2005) were identified in RSV-transformed chicken embryonic fibroblasts (CEF) as the first non-mammalian cytokine. Subsequently, another chicken homologue, K60 (also referred to as chCXCLi1) whose share 67% amino acid sequence identity with chCXCLi2 was identified in lipopolysaccharide (LPS)-induced chicken macrophage cell line (Sick et al., 2000). With high sequence homology with huCXCL8 (48% and 46.5% for chCXCLi1 and chCXCLi2, respectively), adjacent genomic location on chicken chromosome 4 and sharing chemokine receptor chCXCR1, it has suggested that they have evolved by gene duplication (Kaiser et al., 1999, 2005; Poh et al., 2008). Similar to huCXCL8, cxCXCLi2 has chemotactic activity, mainly attracting monocytes, while huCXCL8 is the major chemokine necessary for attracting neutrophils (Barker et al., 1993; Martins-Green and Feugate, 1998). Moreover, chCXCLi2 is involved in angiogenesis and fibroblast mitogenesis (Barker and Hanafusa, 1990). Unlike chCXCLi2, the major type of cells migrated by chCXCLi1 was heterophils, avian equivalent to mammalian neutrophils suggesting the different role of chicken CXCL8 homologues in immune system (Poh et al., 2008).

Although use of monoclonal antibodies (mAbs) has become routine in mammalian research and diagnosis, detecting and quantifying bioactive chemokines/cytokines in avian species is limited by the lack of specific antibodies and reliable bioassays.

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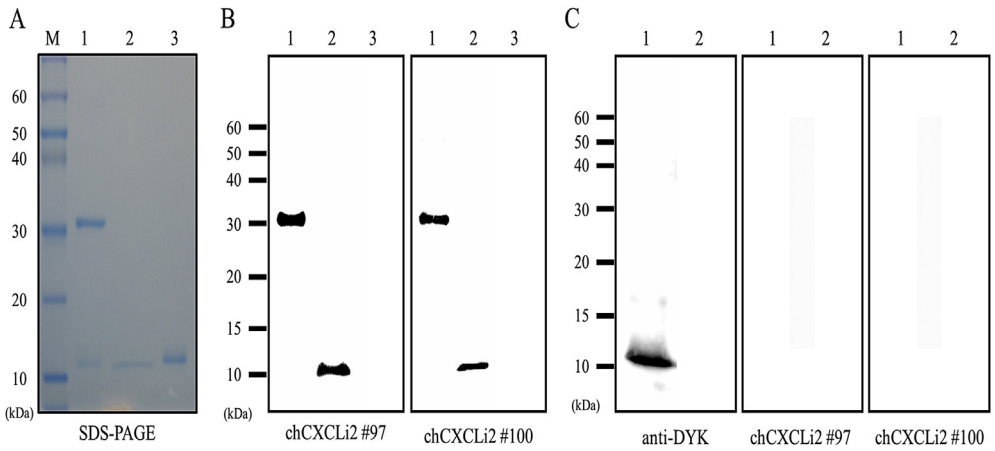


Fig. 1. Recombinant chCXCLi2 determination. (A) Purified recombinant *E. coli* chCXCLi2 (lane 1), yeast chCXCLi2 (lane 2), and yeast chIL-16 (lane 3) were resolved by SDS-PAGE and stained with Coomassie blue. (B) Western blot analysis of samples blotted using chCXCLi2 #97 (B, left panel) and chCXCLi2 #100 (B, right panel). (C) Cross-reactivity of chCXCLi2 mAbs with chCXCLi1. Collected supernatant (lane 1) and cell lysate (lane 2) of COS-7 cells transfected pcDNA3.1/chCXCLi1-DYK was blotted with rabbit anti-DYKDDDL tag antibody (left panel), chCXCLi2 #97 (middle panel) and chCXCLi2 #100 (right panel). Molecular weight (kDa) marker is indicated on the left. Data represent two independent experiments.

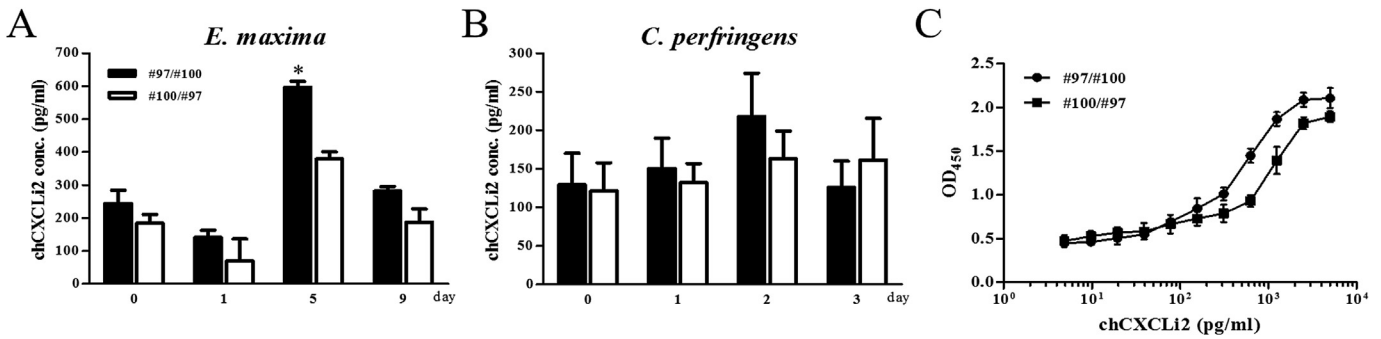


Fig. 2. Endogenous chCXCLi2 determination. Antigen capture assay to detect serum chCXCLi2 in normal (0 day), *E. maxima*- (A), and NE-infected chickens (B). The black bar indicates chCXCLi2 #97 used as capture antibody and chCXCLi2 #100 used as detection antibody (#97/#100) and the white bar indicates chCXCLi2 #100 used as capture antibody and chCXCLi2 #97 used as detection antibody (#100/#97). Data represent the mean ± SD of triplicate assays from two independent experiments. $^{*}p < 0.05$, as compare to 0 day control chickens. (C) Standard curve of chCXCLi2 ELISA using yeast chCXCLi2. Data represent the mean ± SD of triplicate assays from two independent experiments.

Therefore, this study was undertaken to develop mAbs against chCXCLi2, to characterize their ability to neutralize chicken macrophage cell line (HD11) proliferation, and to enable their use as reagents for basic and applied poultry research. Unfortunately, we could not detect cross-reactivity of mAbs between chCXCLi1 and cxCXCLi2 in any assays used in this study.

2. Materials and methods

2.1. Recombinant chCXCLi2 production

In order to produce antigens for generating mAbs, we used recombinant chCXCLi2 protein expressed in *Escherichia coli*. The cDNA encoding chCXCLi2 (GenBank accession: NM_205498) was cloned into the pET32a bacterial expression vector (Novagen, Madison, WI, USA) incorporating an NH2-terminal polyhistidine epitope tag using the following primers: 5′–GATCGGATCCGCTCTGTCGCAAGGTAGGA–3′ and 5′–GATCAAGCTTCACGTGGTGCATCAGAAT–3'. Primers contained BamHI and HindIII restriction enzyme sites (underlined). Recombinant chCXCLi2/pET32a was transformed into *E. coli* BL21(DE3) cells (Life Technologies, Grand Island, NY, USA) and induced in 2X TY liquid culture medium (16 g/L BD Bacto Tryptone (BD Biosciences, San Jose, CA, USA), 10 g/L yeast extract, 6 g/L NaCl) with 0.5 mM isopropyl-1-thio-β-d-galatopyranoside during the mid-log phase (OD_{600} = 0.4) at 37 °C for 3 h. The recombinant chCXCLi2 expressed in *E. coli* (*E. coli* chCXCLi2) fusion protein was purified using Ni_2^+-NTA His•bind Resin (Merck Millipore, Billerica, MA, USA) column chromatography. Another chCXCLi2 protein produced from *Pichia pastoris* (yeast chCXCLi2) for bioactivity assays was obtained from Kingfisher Biotech, Inc. (St. Paul, MN, USA). The concentration of purified *E. coli* chCXCLi2 protein and yeast chCXCLi2 protein were determined using the Bradford assay and purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. To test cross-reactivity of mAbs, another homologue of huCXCL8, chCXCLi1 was cloned into pcDNA3.1 vector (Invitrogen, USA) with DYKDDDDK tag at the C-terminus and produced in COS-7 cells. The collected supernatant from COS-7 cells transfected with pcDNA3.1/chCXCLi1-DYK was concentrated by Amicon ultrafiltration using 3-kDa cutoff filters (Millipore, USA) and used in Western blot analysis with rabbit anti-DYKDDDK tag antibody (Cell signaling, USA) and ELISA to determine the cross-reactivity.

2.2. Production of chCXCLi2 mAb and validation of antigen specificity

All animal protocols were approved by the Beltsville

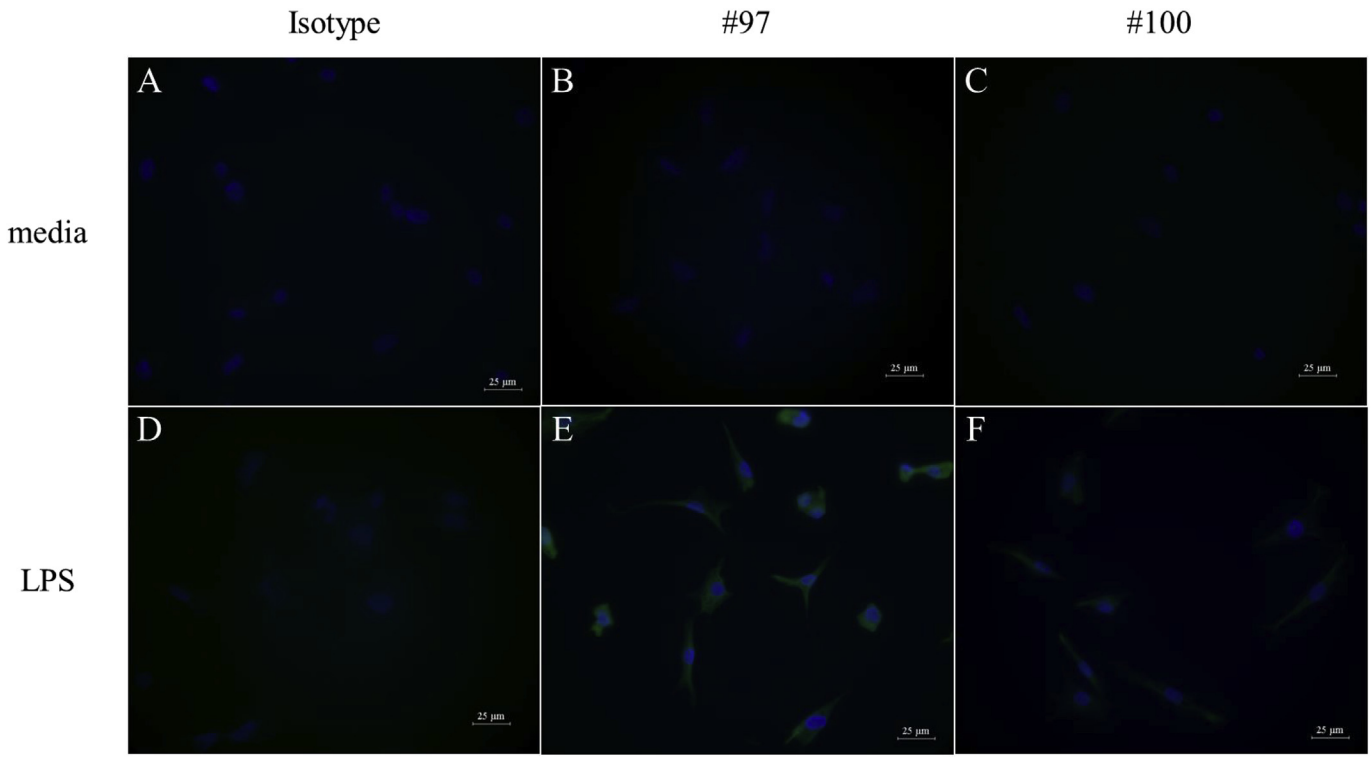


Fig. 3. Immunocytochemistry of chCXCLi2. LPS-treated (10 μg/mL) (D–F) and -untreated CEF cells (A–C) were stained with chCXCLi2 #97 (B and E), chCXCLi2 #100 mAbs (C and F) or isotype control antibody (A and D), and incubated with anti-mouse IgG Alexa 488 secondary antibody (green). DAPI was used to stain the cell nuclei (blue).

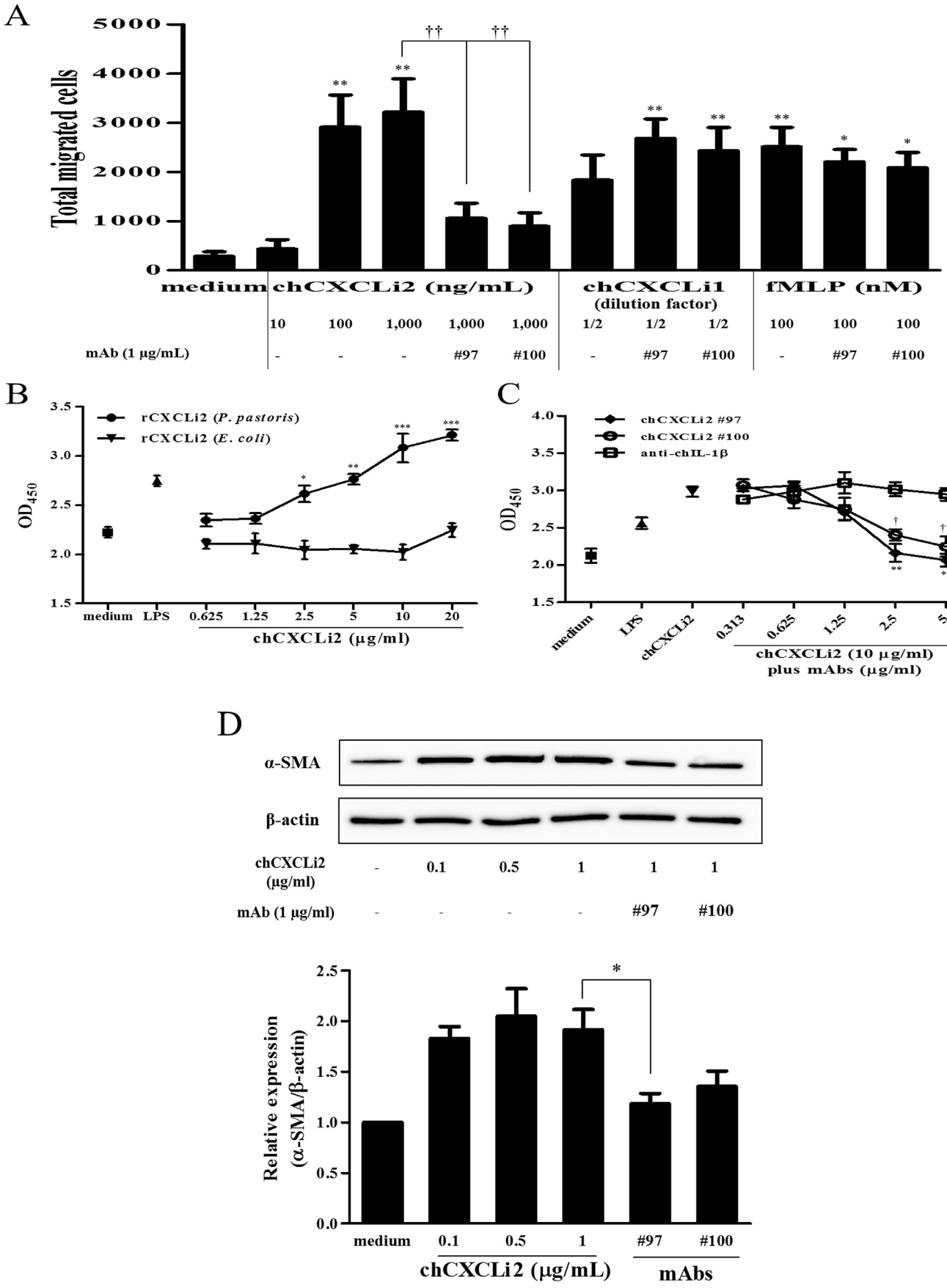
Agricultural Research Center Institutional Animal Care and Use Committee. BALB/c mice (National Cancer Institute, Frederick, MD, USA) were immunized biweekly by intraperitoneal and subcutaneous injections with 50 μg purified *E. coli* chCXCLi2 protein in Freund's adjuvant (Sigma, St. Louis, MO, USA), as previously described (Lee et al., 2011). Booster injections of 25 μg chCXCLi2 without adjuvant were administered intravenously three days prior to cell fusion. Splenic lymphocytes were fused with mouse SP2/0 cells (ATCC, Manassas, VA, USA) and hybridomas selected in RPMI-1640 medium supplemented with hypoxanthine, aminopterin, and thymidine (all from Sigma). Hybridomas secreting chCXCLi2 mAbs were selected by enzyme-linked immunosorbent assay (ELISA), as previously described (Min et al., 2002). Briefly, 96-well microtiter plates were coated overnight at 4 °C with 1.0 μg/well purified *E. coli* CXCLi2 protein. Plates were blocked with phosphate-buffered saline (PBS) containing 1.0% bovine serum albumin and washed with PBS (pH 7.2) containing 0.05% Tween 20 (PBS-T). Undiluted hybridoma culture supernatant (100 μL/well) was added, incubated with agitation at room temperature for 1 h, and washed with PBS-T. A mAb detecting recombinant chIL-10 was used as a negative control. Bound mAbs were detected using horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG secondary Ab (1/1000 dilution), 3,3′,5,5'-tetramethylbenzidine (TMB) substrate, and H_2O_2 (all from Sigma). Optical density at 450 nm (OD_{450}) was determined by a microplate reader (Bio-Rad, Richmond, CA, USA). Hybridoma supernatant containing mAbs that reacted with *E. coli* CXCLi2 only were selected for limiting dilution and further characterization. The monoclonal antibodies were purified from hybridoma supernatant by Protein G agarose chromatography then the purified antibodies were conjugated with HRP by using peroxidase labeling kit (Roche, Germany) according to the manufacturer's instructions. Immunoglobulin isotypes were determined using a mouse mAb isotyping kit (Sigma), according to the manufacturer's instructions.

2.3. Western blot analysis

All samples were mixed with an equal volume of sample buffer (0.125 M Tris–HCl (pH 6.8), 4.0% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue) and heated at 95 °C for 5 min. Each protein (2 μg/lane) was resolved on a 15% SDS-polyacrylamide gel and electroblotted onto nitrocellulose (Immobilon-P, Millipore, Bedford, MA, USA). The membrane was blocked with 1X PBS (pH 7.2) containing 5.0% non-fat dry milk, washed with 1X PBS-T, and incubated with mAb chCXCLi2 #97 and chCXCLi2 #100 (1.0 μg/mL). Bound mAbs were incubated with HRP-conjugated rabbit anti-mouse IgG secondary Ab (1/1000 dilution), visualized using a Clarity Western ECL Substrate (Bio-Rad), and detected using the ChemiDoc imaging system (Bio-Rad).

2.4. Capture ELISA for determining serum chCXCLi2 concentrations

An antigen-capture ELISA was developed using the two mAbs specific for chCXCLi2 and the two antibodies were subsequently used for both capture and detection. The chCXCLi2 #97 or chCXCLi2 #100 used as capture antibodies were coated in carbonate buffer at 0.1 μg/well into 96-well microtiter plates overnight at 4 °C. The plates were blocked and washed, as previously described (Lee et al., 2013). Chicken sera from the control, *Eimeria maxima*-infected, or necrotic enteritis (NE)-infected groups (n = 4/group)—derived by infecting *E. maxima* and *Clostridium perfringens* (Lee et al., 2013)—were diluted 1:2 (v/v) in PBS-T, and a 100 μL of this solution was added to the wells and incubated for 2 h at room temperature. NE was induced as previously described (Park et al., 2008). The plates


A
Total migrated cells
5000
4000
3000
2000
1000
0
medium
chCXCLi2 (ng/mL)
chCXCLi1 (dilution factor)
fMLP (nM)
10 100 1,000 1,000 1,000 1/2 1/2 1/2 100 100 100
mAb (1 μg/mL) - - - #97 #100 - #97 #100 - #97 #100
B
rCXCLi2 (P. pastoris)
rCXCLi2 (E. coli)
OD450
medium LPS 0.625 1.25 2.5 5 10 20
chCXCLi2 (μg/ml)
C
chCXCLi2 #97
chCXCLi2 #100
anti-chIL-1β
OD450
medium LPS chCXCLi2 0.313 0.625 1.25 2.5 5
chCXCLi2 (10 μg/ml) plus mAbs (μg/ml)
D
α-SMA
β-actin
chCXCLi2 (μg/ml) - 0.1 0.5 1 1 1
mAb (1 μg/ml) - - - - #97 #100
Relative expression (α-SMA/β-actin)
medium 0.1 0.5 1 #97 #100
chCXCLi2 (μg/mL)
mAbs

were washed with PBS-T, and a 100 μL/well of peroxidase-conjugated chCXCLi2 #100 or chCXCLi2 #97 (1 μg/mL) detection antibody was added. The plates were incubated for 30 min and then developed with TMB substrate. Optical densities at 450 nm were measured and serum chCXCLi2 concentrations determined using a standard curve generated with known *E. coli* CXCLi2 concentrations.

2.5. Immunostaining of chCXCLi2

CEF cells grown on glass coverslips were stimulated with 10 μg/mL LPS in the presence of GolgiPlug (BD Bioscience) for 2 h. The cells were fixed in 2% paraformaldehyde (Sigma) for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 15 min, blocked in 10% normal horse serum for 20 min, and incubated with chCXCLi2 #97 or chCXCLi2 #100 (10 μg/mL) for 1 h at room temperature. After washing three times with 0.1% Tween 20 in PBS, the coverslips were incubated with anti-mouse IgG Alexa 488 secondary antibodies (Life Technologies) for 1 h. After three washes with 0.1% Tween 20 in PBS, the coverslips were air dried and mounted using Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies) and examined using an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan), using a fixed shutter speed to allow for fluorescence intensity comparison.

2.6. Chemotaxis assay

The chemotaxis of peripheral blood mononuclear cells (PBMC) was determined using ChemoTx 96-well disposable chamber (3 μm pore size filter) (Neuroprobe Inc., Gaithersburg, MD, USA) according to the manufacturer's instruction. PBMC were prepared from 2-weeks old healthy chickens by density gradient methods (Kim et al., 2014) and viability was ≥98%. Recombinant chCXCLi2 or fMLP (Sigma) as a positive control were added to the lower chamber, and isolated PBMC (1×10^5/mL) were suspended in RPMI-1640 supplemented with 1% FBS (HyClone, USA) and 1% Penicillin/Streptomycin (Sigma) and seeded in the upper compartment. Then chamber was incubated for 2 h at 41 °C, 5% CO_2. The cells migrated into the bottom chamber were counted by Cellometer X2 cytometer (Nexcelom Bioscience, Lawrence, MA, USA). Each sample assayed in triplicate and pooled for counting.

2.7. Neutralization of chCXCLi2 by chCXCLi2 mAbs

To identify chCXCLi2 mAbs inhibition of HD11 proliferation, the HD11 cells (5×10^6/mL) were incubated with medium, 10 ng/mL yeast chCXCLi2, or *E. coli* chCXCLi2 pretreated with chCXCLi2 mAbs at the indicated concentrations at 41 °C for 48 h. Cell proliferation was measured using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA) at OD_{450}, as previously described (Lee et al., 2011). To confirm chCXCLi2 mAbs neutralization, CEF cells were incubated with yeast chCXCLi2 at ranges between 312.5 ng/mL and 5000 ng/mL and chCXCLi2, chCXCLi2 #97, and chCXCLi2 #100 mAbs at 41 °C for 24 h. Cell were lysed into radioimmunoprecipitation assay buffer and centrifuged at 15,000 × g for 20 min. The supernatant was mixed with sample buffer and heated at 95 °C for 5 min. Western blot analysis was performed, as previously described, using mouse anti-α-SMA antibody (1A4, Thermo Scientific, Waltham, MA, USA) or rabbit anti-β-actin antibody (Cell Signaling Technology, Danvers, MA, USA) as a loading control.

2.8. Statistical analysis

Each sample was analyzed in triplicate using an ELISA and a cell proliferation assay. All data were subjected to one-way analysis of variance using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA) and were expressed as mean ± SD values. The differences in mean values between control and samples treated with different concentrations of chCXCLi2 or chCXCLi2 mAbs were analyzed using the *t*-test and variations considered significant at $p < 0.05$.

3. Results

3.1. Production of chCXCLi2 mAbs

Two mouse hybridomas (chCXCLi2 #97 and chCXCLi2 #100) secreting mAbs specific for chCXCLi2 protein were identified and cloned based on their strong ELISA reactivity to *E. coli* and yeast chCXCLi2. Isotype analysis of mAbs secreted from these clones revealed that both chCXCLi2 mAbs were IgG1. SDS-PAGE analysis described the specific size of chCXCLi2 and chIL-16 proteins and chCXCLi2 antigen purity for generating mAbs (Fig. 1A). Both mAbs recognize around 30 kDa protein produced in *E.coli* (Fig. 1B, lane 1), which corresponded to the predicted molecular weight of a fusion protein with epitope tag encoded by the pET32a vector, and around 10 kDa protein expressed as a yeast chCXCLi2 (Fig. 1B, lane 2). Neither mAbs reacted with chCXCLi1 or chIL-16 (Fig. 1B and C), suggesting that their binding activity was specific for chCXCLi2. chCXCLi1 is the another chicken homologue of human CXCL8 with sequence homology of 48% and the chCXCLi1-DYK produced in COS-7 cells was detected in supernatant by using anti-DYKDDDK antibody with expected size of 12 kDa. However, both chCXCLi2 mAbs we develop could not detect chCXCLi1 although it has 67% sequence homology with chCXCLi2 (Fig. 1C).

3.2. Serum chCXCLi2 concentration determination

Lymphocytes from *E. maxima*-infected chickens produce high levels of chCXCLi2 transcript (Hong et al., 2006a; 2006b) and *C. perfringens*-induced NE upregulates chCXCLi2 expression in intestinal tissue (Park et al., 2008). Thus, higher serum chCXCLi2 mRNA levels are expected in *E. maxima*- and NE-infected chickens

Fig. 4. Neutralization of chCXCLi2 by chCXCLi2 mAbs. (A) Neutrailzing effect of chCXCLi2 on chemotaxis of chicken PBMC. Chemotaxis assay was performed using ChemoTx system and cell number was counted by automated cytometer. Recombinant chCXCLi1 was produced in COS-7 cells, concentrated and diluted with cell culture medium. fMLP was used as a positive control. Data represent the mean ± SD of duplicate assays from two independent experiments. $^*p < 0.05$ or $^{**}p < 0.01$, as compare to medium control and $\dagger\dagger p < 0.05$, as compare to sample treated with 1000 ng/mL of yeast chCXCLi2. (B) The yeast chCXCLi2-induced proliferation of HD11 chicken macrophage cells. HD11 cells (5×10^6/mL) were incubated with medium alone as a negative control (NC), 10 μg/mL LPS as a positive control, or the indicated concentration of chCXCLi2 protein for 48 h. Data represent the mean ± SD of triplicate assays from two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$, as compare to medium control (C) Neutralizing effect of chCXCLi2 mAbs on HD11 proliferation. HD11 cells (5×10^6/mL) were incubated with medium alone (NC), 10 μg/mL LPS as a positive control, or yeast chCXCLi2 protein (10 μg/mL) with the indicated mAbs concentration for 48 h. Cell proliferation was measured at OD_{450} using WST-8. Data represent the mean ± SD of triplicate assays from two independent experiments. $^{**}p < 0.01$, $\dagger p < 0.05$ or $\dagger\dagger p < 0.01$ as compare to sample treated with 10 μg/mL chCXCLi2. (D) Neutralizing effect of chCXCLi2 mAbs on fibroblast differentiation into myofibroblasts. Cell lysate from yeast chCXCLi2-treated or -untreated CEF cells were separated using SDS-PAGE and analyzed by Western blot using α-SMA (C, upper panel) or β-actin (C, lower panel) as a loading control. Lower panel shows densitometric analysis represent the mean ± SD from two independent experiments. $^*p < 0.05$, as compare to sample treated with 2 μg/mL chCXCLi2.

relative to uninfected controls. The absolute quantification of chCXCLi2 was performed based on standard curves with yeast chCXCLi2 (Fig. 2C). Results from the capture assay using both chCXCLi2 mAbs indicated that serum chCXCLi2 concentrations increased in *Eimeria*- and NE-infected chickens relative to uninfected controls. On day five following *E. maxima* infection, serum chCXCLi2 protein concentrations reached 600 pg/mL ($p < 0.05$) (Fig. 2A). Compared to *E. maxima* infection, NE-infected chickens displayed lower chCXCLi2 serum levels throughout infection (Fig. 2B). Importantly, similar patterns were observed in the ELISA results, using either chCXCLi2 #97 or chCXCLi2 #100 as the capture antibody. These results indicate that both chCXCLi2 mAbs are capable of recognizing endogenous chCXCLi2 in serum and can be used as both capture and detection antibodies.

3.3. Immunostaining of chCXCLi2 by chCXCLi2 mAbs

Binding of chCXCLi2 mAbs to endogenous chCXCLi2 was evaluated using immunocytochemistry. Since LPS treatment induces chCXCLi2 mRNA expression in CEF cells (Barker and Hanafusa, 1990), we used this method to investigate chCXCLi2 detection by chCXCLi2 mAbs. Immunocytochemical study using both mAbs revealed that fluorescence-positive cells were detected in LPS-treated CEF cultures, with specific staining for chCXCLi2 localized to the cytoplasm (Fig. 3).

3.4. Neutralization by chCXCLi2 mAbs

The main role of chemokines is to attract the lymphocytes to inflamed tissues. chCXCLi2 highly induce chemotaxis of PBMC at concentration of 500 ng/mL (Barker et al., 1993). To determine the ability of CXCLi2 mAbs to neutralize the chemotactic activity, Boyden-chamber-based chemotaxis assay was performed. In our study, yeast chCXCLi2 showed highest migration of PBMC at concentration of 1 μg/mL (Fig. 4A). It also shows the migration of PBMC induced by COS-7 cell supernatant containing chCXCLi1. This chemotactic activity of chCXCLi2 was abolished in presence of chCXCLi2 mAbs, #97 or #100 but no inhibition was detected in response to chCXCLi1 or fMLP. It is indicated that the chCXCLi2 mAbs neutralize the ability of yeast chCXCLi2 to attract the lymphocytes into the inflamed tissues. Moreover, yeast chCXCLi2 induced HD11 proliferation in a dose-dependent manner as consistent with chemotaxis (Fig. 4B). The proliferation of HD11 induced by chCXCLi2 was measured by a WST assay to support their neutralizing activity. The yeast chCXCLi2-stimulated HD11 cells cultured in the presence of both chCXCLi2 mAbs exhibited suppressed proliferation in a dose-dependent manner relative to untreated cells whereas mAb against chicken interleukin-1β showed no inhibition of proliferation in any dose uesd (Lee et al., 2014) (Fig. 4C). Although *E. coli* CXCLi2 was used for mAb development, no bioactivity was identified throughout the study. It seems like that the inactivity of bacterially produced chCXCLi2 may attributed by unremoved fusion protein such as tag protein or incomplete folding of recombinants from bacterial system. Additionally, chCXCLi2 mAbs inhibition of CEF myofibroblast differentiation was investigated by measuring myofibroblast marker expression using Western blot analysis. IL-8 induces myofibroblast differentiation in the presence of elevated α-SMA concentrations (Gharaee-Kermani et al., 2012). Given that chCXCLi2 stimulation also produces high α-SMA expression levels (Feugate et al., 2002), we treated yeast chCXCLi2-rich CEF cells with an α-SMA-specific antibody and revealed that chCXCLi2 inhibition by treatment with both antibodies abrogated increased α-SMA expression (Fig. 4D). These results suggest that chCXCLi2 mAbs are capable of inhibiting fibroblast differentiation into myofibroblasts.

4. Discussion

In this study, we described two new mAbs, chCXCLi2 #97 and chCXCLi2 #100, developed against chCXCLi2 with no cross-reactivity with chCXCLi1, which can be used to detect endogenous chCXCLi2 using capture ELISA and allow specific measurement of protein levels for the first time in chickens. Although high homology between chCXCLi1 and chCXCLi2, both mAbs that were developed here did not cross-react with chCXCLi1. Regarding this phenomenon, we speculated that chickens might use different site to interact with its receptors. In humans, it has been known that CXCL8 signaling is mediated by binding of chemokine to two CXC chemokine receptors, CXCR1 and CXCR2 and that the C-X-C motif in CXCL8 is important role in activation of its receptors (Joseph et al., 2010). The avian species like chickens have some degree of similarity in chemokine binding patterns including the binding of chCXCLi1 and chCXCLi2 with chCXCR1 (Poh et al., 2008). Unfortunately, the complete sequence of chicken CXCR2 homologue has not elucidated yet. Both mAbs successfully detected chCXCLi2 in Western blot and immunocytochemistry assays. Furthermore, these mAbs are capable of neutralizing chCXCLi2-induced chemotaxis of PBMC, proliferation of chicken macrophages and differentiation of fibroblasts into myofibroblasts. Interestingly, beside of chemotactic property of chCXCLi2 it has found that chCXCLi2 has diverse role depending on the type of responder cells as PBMC, HD11 and CEF cells were used for assessment of chemotaxis, proliferation and SMA expression, respectively. So far, it has not discovered if chCXCLi2 uses different receptor set for multiple activities but it would be interesting to investigate in further studies. We expect that these new chCXCLi2 mAbs will serve as valuable immune reagents for basic and applied research in poultry.

Acknowledgments

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References

Barker, K.A., Hanafusa, H., 1990. Expression of 9E3 mRNA is associated with mitogenicity, phosphorylation, and morphological alteration in chicken embryo fibroblasts. Mol. Cell. Biol. 10, 3813–3817.
Barker, K.A., Hampe, A., Stoeckle, M.Y., Hanafusa, H., 1993. Transformation-associated cytokine 9E3/CEF4 is chemotactic for chicken peripheral blood mononuclear cells. J. Virol. 67, 3528–3533.
Bedard, P.A., Alcorta, D., Simmons, D.L., Luk, K.C., Erikson, R.L., 1987. Constitutive expression of a gene encoding a polypeptide homologous to biologically active human platelet protein in Rous sarcoma virus-transformed fibroblasts. Proc. Natl. Acad. Sci. U. S. A. 84, 6715–6719.
Bickel, M., 1993. The role of interleukin-8 in inflammation and mechanisms of regulation. J. Periodontol. 64, 456–460.
Fernandez, E.J., Lolis, E., 2002. Structure, function, and inhibition of chemokines. Annu. Rev. Pharmacol. Toxicol. 42, 469–499.
Feugate, J.E., Li, Q., Wong, L., Martins-Green, M., 2002. The cxc chemokine cCAF stimulates differentiation of fibroblasts into myofibroblasts and accelerates wound closure. J. Cell Biol. 156, 161–172.
Gharaee-Kermani, M., Kasina, S., Moore, B.B., Thomas, D., Mehra, R., Macoska, J.A., 2012. CXC-type chemokines promote myofibroblast phenoconversion and prostatic fibrosis. PLoS One 7, e49278.
Heidemann, J., Ogawa, H., Dwinell, M.B., Pafiee, P., Maaser, C., Gockel, H.R., Otterson, M.F., Ota, D.M., Lűgering, N., Domschke, W., Binion, D.G., 2003. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. J. Biol. Chem. 278, 8508–8515.
Hong, Y.H., Lillehoj, H.S., Lee, S.H., Dalloul, R. a., Lillehoj, E.P., 2006a. Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. Vet. Immunol. Immunopathol. 114, 209–223.
Hong, Y.H., Lillehoj, H.S., Lillehoj, E.P., Lee, S.H., 2006b. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens. Vet. Immunol. Immunopathol. 114, 259–272.
Joseph, P.R., Sarmiento, J.M., Mishra, A.K., Das, S.T., Garofalo, R.P., Navarro, J.,

Rajarathnam, K., 2010. Probing the role of CXC motif in chemokine CXCL8 for high affinity binding and activation of CXCR1 and CXCR2 receptors. J. Biol. Chem. 285, 29262–29269.
Kaiser, P., Hughes, S., Bumstead, N., 1999. The chicken 9E3/CEF4 CXC chemokine is the avian orthologue of IL8 and maps to chicken chromosome 4 syntenic with genes flanking the mammalian chemokine cluster. Immunogenetics 49, 673–684.
Kaiser, P., Poh, T.Y., Rothwell, L., Avery, S., Balu, S., Pathania, U.S., Hughes, S., Goodchild, M., Morrell, S., Watson, M., Bumstead, N., Kaufman, J., Young, J.R., 2005. A genomic analysis of chicken cytokines and chemokines. J. Interferon Cytokine Res. 25, 467–484.
Kim, W.H., Jeong, J., Park, A.R., Yim, D., Kim, S., Chang, H.H., Yang, S.H., Kim, D.H., Lillehoj, H.S., Min, W., 2014. Downregulation of chicken interleukin-17 receptor A during *Eimeria* infection. Infect. Immun. 82, 3845–3854.
Lee, S.H., Lillehoj, H.S., Jang, S.I., Baldwin, C., Tompkins, D., Wagner, B., Parcells, M., Del Cacho, E., Hong, Y.H., Min, W., Lillehoj, E.P., 2011. Development and characterization of mouse monoclonal antibodies reactive with chicken interleukin-2 receptor αlpha chain (CD25). Vet. Immunol. Immunopathol. 144, 396–404.
Lee, S.H., Lillehoj, H.S., Jang, S.I., Lillehoj, E.P., Min, W., Bravo, D.M., 2013. Dietary supplementation of young broiler chickens with *Capsicum* and turmeric oleoresins increases resistance to necrotic enteritis. Br. J. Nutr. 110, 840–847.
Lee, S.H., Lillehoj, H.S., Jeong, M.S., Del Cacho, E., Kim, J.B., Kim, H.R., Min, W., Jeoung, H.Y., An, D.J., 2014. Development and charaterization of mouse monoclonal antibodies reactive with chicken IL-1β. Poult. Sci. 93, 2193–2198.
Martins-Green, M., Feugate, J.E., 1998. The 9E3/CEF4 gene product is a chemotactic and angiogenic factor that can initiate the wound-healing cascade in vivo. Cytokine 10, 522–535.
Min, W., Lillehoj, H.S., Li, G., Sohn, E.J., Miyamoto, T., 2002. Development and characterization of monoclonal antibodies to chicken interleukin-15. Vet. Immunol. Immunopathol. 88, 49–56.
Modi, W.S., Dean, M., Seuanez, H.N., Mukaida, N., Matsushima, K., O'Brien, S.J., 1990. Monocyte-derived neutrophil chemotactic factor (MDNCF/IL-8) resides in a gene cluster along with several other members of the platelet factor 4 gene superfamily. Hum. Genet. 84, 185–187.
Park, S.S., Lillehoj, H.S., Allen, P.C., Park, D.W., FitzCoy, S., Bautista, D., Lillehoje, E.P., 2008. Immunopathology and cytokine responses in broiler chickens coinfected with *Eimeria maxima* and *Clostridium perfringens* with the use of an animal model of necrotic enteritis. Avian Dis. 52, 14–22.
Poh, T.Y., Pease, J., Young, J.R., Bumstead, N., Kaiser, P., 2008. Re-evaluation of chicken CXCR1 determines the true gene structure. J. Biol. Chem. 283, 16408–16415.
Sugano, S., Stoeckle, M.Y., Hanafusa, H., 1987. Transformation by Rous sarcoma virus induces a novel gene with homology to a mitogenic platelet protein. Cell 49, 321–328.
Sick, C., Schneider, K., Staeheli, P., Weining, K.C., 2000. Novel chicken CXC and CC chemokines. Cytokine 12, 181–186.